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Partial Reactions of Photosynthesis in Briefly Sonicated *Chlamydomonas*

I. CELL BREAKAGE AND ELECTRON TRANSPORT ACTIVITIES[1]

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ABSTRACT

The cell structure of *Chlamydomonas reinhardi* is disrupted by brief exposure to sonication. The extent of cell breakage can be determined quickly by cell count with the light microscope. Rates of photochemical activities of briefly sonicated cells approach those reported for higher plant chloroplasts. These activities are a sensitive function of time of sonication and sonic power used. The method of brief sonication is rapid and convenient and gives a stable preparation useful for determining photochemical activities in *Chlamydomonas*.

The green alga, *Chlamydomonas reinhardi*, has become an increasingly useful organism for research in mutational analysis of photosynthetic mechanisms, development studies of chloroplasts, and genetic and molecular biological investigations of nuclear and cytoplasmic inheritance (8–11). In many of these studies, the measurement of partial reactions of photosynthesis (or photochemical activities) is of crucial importance. For example, because Levine and co-workers were able to measure photochemical activities in particles of *Chlamydomonas reinhardi*, they could demonstrate that several single gene mutations affected uniquely these activities (9).

The majority of past studies of photochemical activities in algae has involved some kind of mechanical breakage of the cells followed by differential centrifugation to isolate photosynthetic membranes. The tough algal cell wall requires rather vigorous treatment to liberate the photosynthetic membranes such that the isolated chloroplast membranes often exhibit rather poor photochemical activities compared to those from higher plant chloroplasts.

In this and the following paper (3), we demonstrate the value of using a briefly sonicated cell preparation in studying the photochemical activities in *Chlamydomonas reinhardi*.

MATERIALS AND METHODS

Chlamydomonas reinhardi wild type strain 137-C (+) was grown in 500-ml flasks as described by Gorman and Levine (5). Cells were harvested during log phase growth by centrifugation at 3000*g* for 5 min. They were suspended in 0.05 M Tricine, pH 7.5, and again pelleted under the same centrifugation conditions. Cells were then resuspended in 0.05 M Tricine, pH 7.5, at a concentration of 0.06 mg Chl/ml, and the suspension was slowly stirred at room temperature under a fluorescent lamp which provided an incident light intensity of 1×10^4 ergs cm^{-2} sec^{-1}. In assays where pH values considerably different from 7.5 were used, cells were resuspended at a concentration of 0.01 M Tricine, pH 7.5. Chlorophyll was determined by the method of Arnon (1).

Cells were sonicated in preparation for assay in the following manner. After the harvested cells had been illuminated for at least 15 min under the fluorescent lamp as described above, a 5-ml aliquot was placed in a 10-ml glass beaker. Sonication was performed with a Quigley-Rochester sonic dismembrator equipped with a 1.27-cm diameter probe. The probe was placed approximately two-thirds of the way into the cell suspension. The power unit of the sonicator was equipped with a dial to permit power settings calibrated between 0 and 100, and connected to a timer such that sonication times of 2 sec or more could be used with accuracy. An adjustable laboratory jack was placed below the sonicator probe to support the beaker of cells at a precise and constant position with respect to the probe. No attempt was made to control the temperature during sonication. Immediately after sonication aliquots were removed for assays.

Assays of O_2 evolution or uptake were performed with a Y.S.I. Model 53 oxygen monitor equipped with a Clark electrode. Illumination was provided by a tungsten lamp (1×10^6 ergs cm^{-2} sec^{-1}). The jacketed electrode chamber contained 1.3 ml of volume and was kept at 25 C. Rates were determined after 45 sec preillumination to avoid transient effects of light on the electrode.

NADP photoreduction was performed with a Hitachi/Perkin-Elmer Model 124 spectrophotometer. The cuvette chamber was modified to permit continuous stirring of the sample with a magnetic stirrer located directly below the sample cuvette holder. A microscope lamp was mounted below the cuvette chamber to permit continuous illumination (from an appropriate mirror arrangement) of the sample from the side and at right angles to the measuring beam. The photomultiplier was isolated from the actinic light by placing a Corning filter No. 2-73 between the actinic source and the cuvette and a Dell Optics interference filter (3400 Å transmission peak, 45% transmission maximum, half-band width of 150 Å) between the cuvette and the photomultiplier. The actinic light was isolated from the reference cuvette chamber and a neutral density filter placed in the reference chamber to facilitate

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balancing of the beams. Actinic light intensity at the cuvette was 1×10^5 ergs cm^{-2} sec^{-1}. NADP reduction was monitored continuously at 340 nm with a strip chart recorder attached to the spectrophotometer.

Cells were observed before and after various times of sonication with an American Optical Corp. Series 10 phase microscope.

MES-Tricine buffer was prepared in the following manner:

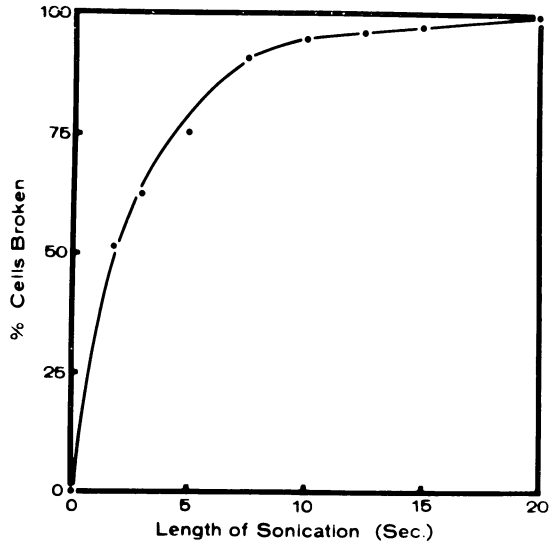

FIG. 1. Cell breakage by sonication. Cells were sonicated at a power setting of 80. Whole cells remaining after sonication were counted using a hemacytometer.

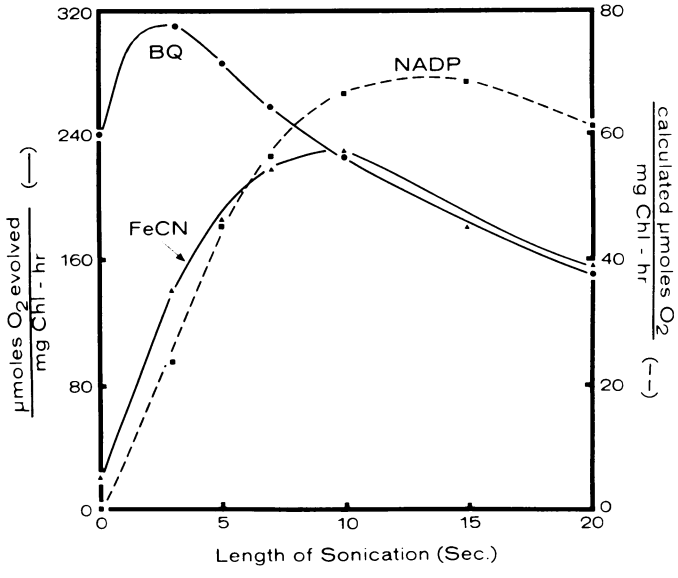

FIG. 2. Effect of sonication time on O_2-evolving reactions. Cells were sonicated at a power setting of 80. Each reaction contained sonicated cells equivalent to 30 μg of Chl. Ferricyanide reduction reactions (▲) contained 50 μmoles of MES-Tricine buffer (pH 8.1), 1.5 μmoles of $K_3Fe(CN)_6$, and 10 of μmoles MeAm in a final volume of 1.3 ml of BQ reduction reactions (●) contained 50 μmoles of MES-Tricine buffer (pH 8.4), 0.75 μmole of BQ, and 10 μmoles of MeAm in a final volume of 1.3 ml. NADP reduction reactions (■) contained 50 μmoles of MES-Tricine (pH 7.5), 30 nmoles of spinach ferredoxin, 0.5 μmole of NADP, and 15.4 of μmoles MeAm in a final volume of 2.0 ml. Rate of NADP-mediated O_2 evolution was calculated by dividing the rate of NADPH formation by 2. Note that there are two ordinates.

0.5 M MES was titrated to pH 5.4 and 0.5 M Tricine was titrated to pH 9.0 with NaOH. These two solutions were then mixed in the appropriate proportions to obtain the desired pH.

NADP, DPIP,[2] and Tricine and MES buffers were obtained from Sigma Chemical Co. *p*-Benzoquinone (practical grade) was obtained from Matheson, Coleman and Bell. Methyl viologen was purchased from Mann Research Laboratories and sodium ascorbate from Calbiochem. All other chemicals were reagent grade. Spinach ferredoxin was prepared as described previously (4).

RESULTS

Previous methods of measurement of specific photochemical activities in *Chlamydomonas reinhardi* and other algal species have been somewhat inconvenient and often resulted in variable or low activities. We find that brief sonication of whole cells with no attempt to isolate chloroplasts or membrane fragments from the broken cells gives particles with photochemical activities comparable to those observable in freshly isolated higher plant chloroplasts.

Chlamydomonas cells are rapidly broken by sonication. One second sonication at a power setting of 80 removed flagella from virtually all cells, although no other change was apparent. However, after 5 sec sonication a large percentage of cells was disrupted, with many completely disintegrated; those remaining appeared swollen and rounded. After 20 sec sonication nearly all cells were broken, with only a few cells still visible at 1500× as viewed under bright field or dark phase.

When viewed at low power (100×) with the light microscope neither the cells which had lost their chloroplast, nor the diffuse membrane material was easily visible. Thus, cell counts in a hemocytometer under low power with the light microscope after various times of sonication could be used to determine the extent of breakage. The results of one such cell count are shown in Figure 1.

Cells were also observed with the electron microscope after glutaraldehyde fixation followed by osmium tetroxide and sectioning (Rudy Turner, personal communication). The extent of breakage noted with the electron microscope was very similar to that in the light microscope. No specific breakage pattern was discernible, and in some cases the internal plastid membrane structure was disrupted while the other cell wall and membrane appeared to remain intact.

The effect of sonication time on various electron transport activities is shown in Figure 2. In each case, the observed rate is maximal after only a few sec of sonication whereas excessive sonication resulted in decreased activity. As has previously been observed (2), O_2 evolution mediated by BQ can proceed in whole cells without mechanical disruption. However, considerably better rates are observed after 3 sec sonication. Ferricyanide-mediated O_2 evolution does not occur in unsonicated cells; yet, after 10 sec of sonication, rates comparable to those obtainable with isolated higher plant chloroplasts are achieved. The Hill reaction mediated by ferredoxin plus NADP could not be accurately measured with the O_2 electrode because of a superimposed Mehler reaction mediated by ferredoxin. Therefore, the rate of NADP reduction was measured spectrophotometrically and the stoichiometric O_2 evolution was calculated therefrom. Comparing the three curves in Figure 2, it appears that longer sonication times are required for the functioning of large, hydrophilic acceptors (ferredoxin/NADP) than for small more lipophilic compounds (BQ).

[2]Abbreviations: Asc: sodium ascorbate; BQ: *p*-benzoquinone; DPIP: 2,6-dichlorophenol-indophenol; FeCN: potassium ferricyanide; MeAm: methylamine; MV: methyl viologen.

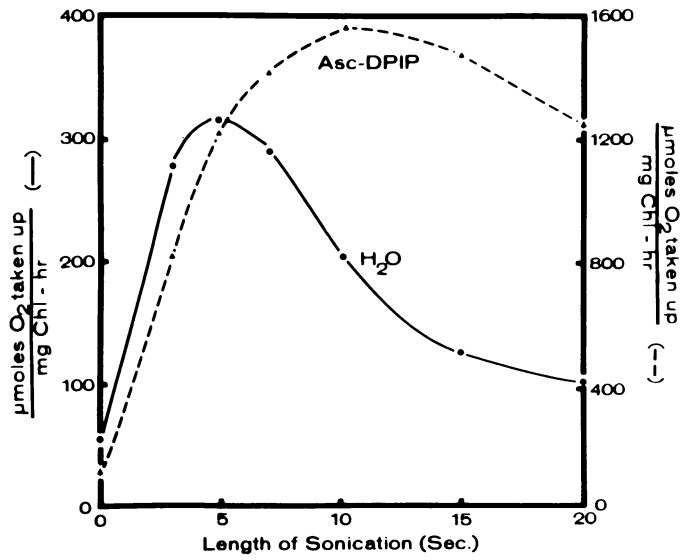

FIG. 3. Effect of sonication time of O_2-uptake reactions. Cells were sonicated at a power setting of 80. Reactions using H_2O as electron donor (●) contained sonicated cells equivalent to 30 μg of Chl, 50 μmoles of MES-Tricine buffer (pH 8.1), 0.1 μmole of MV, 1.0 μmole of KCN, and 10 μmoles of MeAm in a final volume of 1.3 ml. Reactions using ascorbate as electron donor (▲) contained sonicated cells equivalent to 6 μg of Chl, 50 μmoles of MES-Tricine buffer (pH 7.8), 5 μmoles of Asc, 0.05 μmole of DPIP, 0.1 μmole of MV, 0.01 μmole of DCMU, 2 μmoles of KCN, and 10 of μmoles MeAm in a final volume of 1.3 ml. Note that there are two ordinates.

Table I. *Optimum Sonication Power Settings for Electron Transport*
All cells were sonicated 10 sec.

Reaction	Optimum Sonication Power Setting	Rate
		μmoles O_2/mg Chl·hr
$H_2O \rightarrow BQ$[1]	60	212[2]
$H_2O \rightarrow FeCN$[1]	70	125[2]
$H_2O \rightarrow MV$[3]	80	130[4]
$H_2O \rightarrow NADP$[1]	90	62[5]
Asc/DPIP → MV[3]	90–100	1140[4]

[1] Reaction conditions as described in the legend of Fig. 2.
[2] O_2 evolution.
[3] Reaction conditions as described in the legend of Fig. 3.
[4] O_2 taken up.
[5] O_2 evolution as calculated in the legend of Fig. 2.

The data in Figure 3 demonstrate that O_2 consuming reactions in briefly sonicated *Chlamydomonas* cells respond analogously to the O_2 evolving reactions. Photosystem II is apparently more sensitive to sonication than photosystem I since the reaction involving O_2 evolution (H_2O to MV) is about 60% inhibited after 15 sec sonication, while that involving only photosystem I (Asc/DPIP to MV) is nearly optimal after 15 sec sonication (Fig. 3).

Table I shows the optimum sonication power settings for 10 sec sonication. Maximum activities occur at somewhat different power settings for different assays. However, each assay system showed nearly maximum activity with a power setting of 80. To afford maximum uniformity, experiments were rountinely performed with cells sonicated for 10 sec at a power setting of 80.

The pH optima of several photochemical activities are shown in Figure 4. Electron transfer from H_2O to BQ exhibits a broad pH optimum from 7.5 to 8.1. Approximately the same optimum is seen with FeCN or MV as electron acceptor (data not shown). The pH optimum for NADP reduction is lower; the activity is maximal between 6.9 and 7.5. MV reduction mediated by Asc/DPIP demonstrates a pH optimum of 8.4 to 8.7. This assay was usually performed at pH 7.8 or 8.1 because at higher pH the activity was variable and generally increased slowly with time after sonication.

The effects of the photophosphorylation uncoupler methylamine and of salt concentration of photochemical activities are given in Table II. Except for NADP reduction each activity was stimulated about 30% by MeAm.

Both sodium and magnesium chlorides are inhibitory to all activities in the concentration range tested, with Mg inhibiting most severely. However, all activities except NADP could tolerate a moderate amount of salt. NADP reduction was

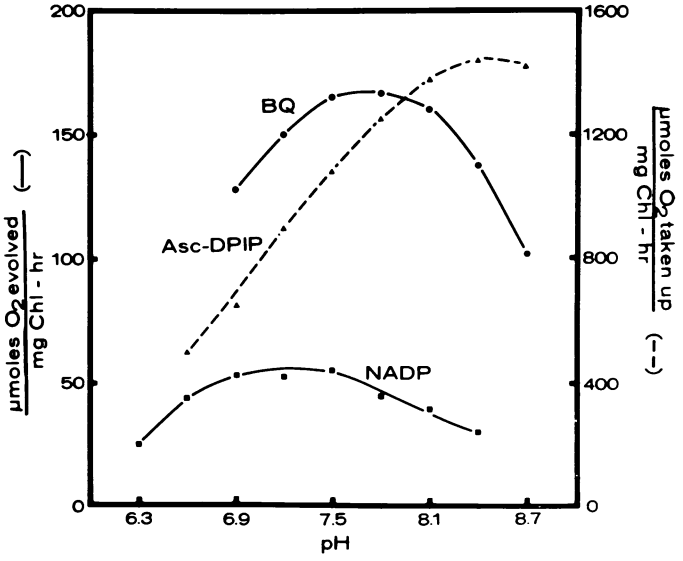

FIG. 4. pH dependence of electron transport reactions. Cells were sonicated 10 sec at a power setting of 80. NADP reduction reactions (■) and BQ reduction reactions (●) were as described in the legend of Figure 2. Reactions with ascorbate as electron donor (▲) were as described in the legend of Fig. 3. Note that there are two ordinates.

Table II. *Effect of Methylamine and Salts on Electron Transport Reactions*
Values are given as percentage of the reaction with methylamine.

Reaction	Additions[1]				
	None	MeAm	MeAm + 0.23 M NaCl	MeAm + 1.2 M NaCl	MeAm + 0.4 M $MgCl_2$
$H_2O \rightarrow BQ$[2]	74	100 (306)[3]	55	26	41
$H_2O \rightarrow FeCN$[2]	77	100 (238)	81	34	45
$H_2O \rightarrow MV$[4]	54	100 (170)	62	26	22
$H_2O \rightarrow NADP$[2]	123	100 (55)	6	0	0
Asc/DPIP → MV[4]	62	100 (1250)	67	40	22

[1] When present, methylamine concentration was 7.7 mM.
[2] Reaction conditions as described in the legend of Fig. 2.
[3] Numbers in parentheses indicate μmoles O_2/mg Chl·hr.
[4] Reaction conditions as described in the legend of Fig. 3.

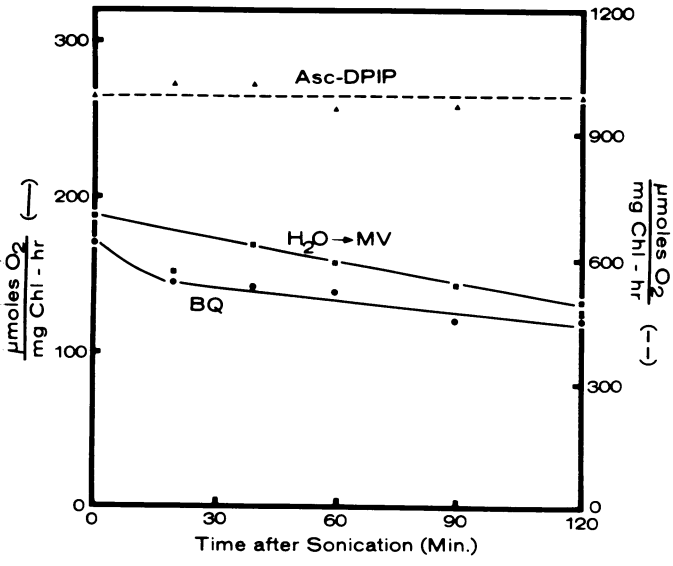

FIG. 5. Stability of sonicated cells. Cells were sonicated 10 sec at a power setting of 80, and maintained at 0 C following sonication. BQ reduction reaction (●) were as described in the legend of Fig. 2. MV reactions from water (■) and from Asc (▲) were as described in Fig. 3. Note that there are two ordinates.

almost completely inhibited by 0.23 M NaCl and activity is 50% inhibited (data not shown) by less than 0.01 M $MgCl_2$.

A particular advantage of the briefly sonicated system is the stability of various photochemical activities after sonication (Fig. 5). It is clear that several activities are stable for 2 or more hr.

Assay component concentrations for optimum activities were investigated and were found to be very similar to those for isolated higher plant chloroplasts. The concentrations cited in the figure and table legends were found to be optimum under the conditions reported here. Thus, it appears that, with the exception of the brief sonication step, conditions in current use for measuring photochemical activities in higher plant chloroplasts can be adapted to the *Chlamydomonas* system.

DISCUSSION

A major problem in studies of photochemical activities in *Chlamydomonas* has been the difficulty of isolating photosynthetic membranes which provide consistently high electron transport activities. This problem has not yet been solved, but has been circumvented by using a brief sonication to disrupt the cells. No attempt was made to isolate chloroplast membrane material from the broken cells since other cell components did not interfere with the activities measured.

It has been reported that sonication results in uncoupling of electron transport from photophosphorylation (6) and removal of plastocyanin from the membranes of isolated spinach chloroplasts (7). However, the 10 sec sonication routinely used here is considerably shorter than that required to uncouple or remove plastocyanin from higher plant chloroplasts. The rather small stimulation by methylamine (Table II) would indicate that these broken cells are largely uncoupled. However, the results reported in the accompanying paper (3) do not support this view since this preparation catalyzes photophosphorylation. None of the photosystem I activities measured required exogenous plastocyanin, although ferredoxin was needed for NADP reduction.

The crucial step, sonication, proved to be remarkably reproducible. Variation in activity from one preparation to the next could usually be attributed to subtle variations in the method of sonication. When variables were all kept to a minimum, repetitive sonications resulted in activities which were reproducible within 10%. Somewhat variable results from day to day were attributed to slight differences among cell populations.

The advances of this system over those used for many preparations of photosynthetic materials are convenience and speed. Cells could be harvested and left stirring for several hours without appreciable change inactivities. Furthermore, cells could be stored for up to 2 hr at 0 C after sonication without appreciable loss in photochemical activities. In most experiments, when assays were performed immediately after sonication, no cooling of the preparation was required for the entire procedure.

We believe that the brief sonication procedure will not only prove convenient for determining photochemical activities in large numbers of cell populations such as potential mutant strains, but may prove very useful in the classroom, whereby cell preparation and photosynthetic assays can be rapidly performed by students.

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